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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

INVENTOR(S)

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Eun-Kyoung	Kim	

☒ *Additional inventors are being named on the____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

FATTY ACID SYNTHASE INHIBITION REDUCES FOOD INTAKE VIA HYPOTHALAMIC AMP-ACTIVATED PROTEIN KINASE

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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☒ Drawing(s) *Number of Sheets* 5 ☐ Other (specify)

☐ Application Data Sheet. See 37 CFR 1.76

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☒ Applicant claims small entity status. See 37 CFR 1.27.

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Respectfully submitted,

SIGNATURE

Date 3/18/04

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Provisonal Application of:)
EUN-KYOUNG KIM, *et al.*)
Filed:March 18, 2004)
For: **FATTY ACID SYNTHASE**)
INHIBITION REDUCES FOOD)
INTAKE VIA HYPOTHALAMIC)
AMP-ACTIVATED PROTEIN)
KINASE

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Dwayne Franklin
General Services Department

Control of Feeding Behavior by Targeting AMPK

Background

Despite significant advances in the understanding of appetite and satiety at molecular levels, practical therapies for weight loss remain elusive. C75, a synthetic fatty acid synthase (FAS) inhibitor identified in U.S. Patent No. 5.981,575 (incorporated herein by reference), causes profound weight loss and anorexia in lean, diet-induced obese (DIO), and genetically obese (ob/ob) mice. As set forth in International Patent Application PCT/US03/03839 describes that, in addition to FAS inhibition, C75 also stimulates carnitine palmitoyltransferase-1 (CPT-1) activity, increasing fatty acid oxidation and ATP levels. Enzymes of the fatty acid metabolic pathways are highly expressed in hypothalamic neurons that regulate feeding behavior (9). Therefore, alterations in fatty acid metabolism may affect neuronal energy flux, which could signal a change in energy status, leading to changes in feeding behavior.

AMPK (AMP-activated protein kinase) is activated by metabolic stresses such as nutrient starvation (10) and ischemia-hypoxia (11), and by physiological processes such as vigorous exercise (12, 13). Specifically, increases in the AMP/ATP ratio, decreases in cellular pH, and increases in the creatine/phosphocreatine ratio are known to activate AMPK via allosteric activation of AMPK by AMP and phosphorylation of AMPK by AMPKK (14-19). Once activated, AMPK switches off ATP-consuming biosynthetic pathways such as fatty acid synthesis, and switches on ATP-generating metabolic pathways such as fatty acid oxidation to preserve ATP levels (20, 21). The central roles of AMPK in both energy sensing and the control of fatty acid metabolism (16, 22) and its regulation by leptin in muscle (23) make it a candidate

metabolic sensor in the hypothalamus to relay changes in metabolism caused by C75 and other compounds.

Summary of the Invention

Applicants have found a means for regulating food intake by administering a compound which targets the activity of AMPK, in particular hypothalamic AMPK.

Discussion of the Invention

As shown in FIG. 5e, C75 and other compounds can affect feeding behavior through a fairly complex mechanism. . Through its ability to inhibit FAS and stimulate CPT-1, C75 may increase ATP levels in hypothalamic neurons, as it does in the periphery (8) and in cortical neurons (35). This change signals a positive energy balance, leading to a decrease in AMPK activity, resulting in a decrease in NPY expression. In fasting, when energy is depleted, AMPK is stimulated, thereby activating the CREB-NPY pathway and food intake. There appears to be relatively little change in the level of phosphorylated hypothalamic AMPK during normal feeding, and a prolonged period of decreased food intake is required before hypothalamic pAMPK levels increase. Hypothalamic AMPK appears responsive to changes in energy status due to C75 treatment or fasting. Thus, AMPK functions as a "fuel sensor" in the CNS, as it does in peripheral tissues such as muscle (23, 39).

The treatment of obesity remains a daunting medical problem. The present study suggests that at least one consequence of C75's actions is the alteration of AMPK activity, supporting that AMPK is a potential therapeutic target for the treatment of

obesity and type 2 diabetes (45). AMPK serves as a master fuel sensor, since C75's effects dominate over fasting-induced cues, and can even reduce food intake in ob/ob mice.

Compounds which either inhibit or stimulate AMPK may be used to regulate food intake. The compositions of the present invention can be presented for administration to humans and other animals in unit dosage forms, such as tablets, capsules, pills, powders, granules, sterile parenteral solutions or suspensions, oral solutions or suspensions, oil in water and water in oil emulsions containing suitable quantities of the compound, suppositories and in fluid suspensions or solutions. As used in this specification, the terms "pharmaceutical diluent" and "pharmaceutical carrier," have the same meaning. For oral administration, either solid or fluid unit dosage forms can be prepared. For preparing solid compositions such as tablets, the compound can be mixed with conventional ingredients such as talc, magnesium stearate, dicalcium phosphate, magnesium aluminum silicate, calcium sulfate, starch, lactose, acacia, methylcellulose and functionally similar materials as pharmaceutical diluents or carriers. Capsules are prepared by mixing the compound with an inert pharmaceutical diluent and filling the mixture into a hard gelatin capsule of appropriate size. Soft gelatin capsules are prepared by machine encapsulation of a slurry of the compound with an acceptable vegetable oil, light liquid petrolatum or other inert oil.

Fluid unit dosage forms or oral administration such as syrups, elixirs, and suspensions can be prepared. The forms can be dissolved in an aqueous vehicle together with sugar, aromatic flavoring agents and preservatives to form a syrup. Suspensions can be prepared with an aqueous vehicle with the aid of a suspending agent such as acacia, tragacanth, methylcellulose and the like.

For parenteral administration fluid unit dosage forms can be prepared utilizing the compound and a sterile vehicle. In preparing solutions the compound can be dissolved in water for injection and filter sterilized before filling into a suitable vial or ampoule and sealing. Adjuvants such as a local anesthetic, preservative and buffering agents can be dissolved in the vehicle. The composition can be frozen after filling into a vial and the water removed under vacuum. The lyophilized powder can then be scaled in the vial and reconstituted prior to use.

The following examples further elucidate, without limiting, the claimed invention.

Examples

Methods

Animals. All animal experiment was done in accordance with guidelines on animal care and use established by the Johns Hopkins University School of Medicine Institutional Animal Care and Use Committee.

Male BALB/c mice (7-9 weeks) were purchased from Charles River Laboratories (and housed in a controlled-light (a12 hr light/12 hr dark cycle) environment (lights on 0200-1400h) and allowed ad libitum access to standard laboratory chow and water. For fasting, food was withdrawn from cage at the onset of the dark cycle for 24 hr, but ad libitum access to water was allowed. Measurement of food intake. Mice were implanted with permanent stainless steel cannulae into the lateral ventricle of the brain 0.6 mm caudal to Bregma, 1.2 mm lateral to the midline, and sunk to a depth of 2.2 mm below the surface of the skull. Implanted mice were housed in individual cages and utilized for i.c.v. and i.p. injections as indicated. C75 dissolved in RPMI1640 (Gibco-BRL), AICAR

(Toronto Research Chemicals Inc) or compound C (46) (FASgen, Inc.) in saline was injected i.c.v., such that desired dose could be administered in a volume of 2.5 µl, while control groups received vehicle only. Injections were done immediately preceding dark onset and food intake measurements were taken at 1 hr (0-1 hr interval), 3 hr (1-3 hr interval), and 24 hr (3-24 hr interval) after dark onset. C75 i.p./AICAR i.c.v. treatment groups were i.p.injected with 5 mg/kg bodyweight C75 dissolved in 200 ml of glucose-free RPMI 1 hr before the dark onset, followed by 3 µg/2.5 µl saline i.c.v. AICAR immediately preceding the dark onset. Control groups received 200 µl of glucose free RPMI 1 hr before lights off and 2.5 µl of saline. Administration of i.p. compound C (10 or 30 mg/kg bodyweight) or C75 (10 mg/kg bodyweight) was followed by food intake measurement at the same times indicated.

Western blot analysis. Hypothalami were dissected using as landmarks the optic chiasm rostrally, and the mammillary bodies caudally to a depth of 2 mm. Dissected hypothalamic and liver tissue were immediately frozen in liquid nitrogen. Tissues were homogenized in 200 µl of lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM sodium pyrophosphate, 50 mM NaF, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.5 mM PMSF, 0.1 mM benzamidine, 50 µg/ml leupeptin, 50 µg/ml soybean trypsin inhibitor). SDS detergent was added to a final 0.2%, and lysates were boiled for 5 min. After the supernatant was harvested, protein concentration was determined by BCA kit (Bio Rad). Phosphorylation of AMPKa was determined on a 4-15% gradient SDS-polyacrylamide gel using anti-phospho-AMPKa (a1 and a2, Thr172) antibody (1:1000, Cell Signaling). Anti-AMPKa antibody (a1 and a2, 1:1000, Cell Signaling) was used as a loading control.

Primary hypothalamic neuron cultures and ATP measurement. Hypothalami were dissected from E17 Sprague-Dawley rats (Harlan), and dissociated by trypsin (0.125%)-DNA (0.001%) solution and trituration as described (35). Cells were plated at 6x104 on poly-D-lysine coated 96 well plates (Corning Inc.) in neurobasal medium supplemented with B27, 0.5 mM L-glutamine, 1% penicillin-streptomycin (Gibco-BRL). To limit nonneuronal cell proliferation, cells were treated with cytosine arabinoside furanoside (1μM) on day 4 after plating and 6-8 days-old cells were assayed for ATP. Hypothalamic neurons were lysed in TE (100 mM Tris-HCl, pH 7.4, 4 mM DTA), and ATP levels were measured within the linear range using the ATP BioLuminescence Kit CLSII (Roche) by following the manufacture's recommendation. Data were analyzed by a Perkin-Elmer Victor2 1420.

RNA preparation and Northern blot analysis. Hypothalamic total RNA was purified using Trizol reagent (Gibco-BRL) and Northern blot analysis using 15 μg of total RNA was performed as previously described (9). RNA was hybridized with random primed 32P-labeled DNA probes made from cloned plasmids of mouse AGRP (Genebank #U89486), human NPY (XM004941), rat CART (U10071), and mouse POMC (AH005319). As a loading control, the probe for mouse GAPDH gene was used at the same blot. The Signals were quantified using an image analyzer (Molecular Dynamics) and Imagequant software.

Immunohistochemistry. Floating brain sections were prepared as previously described (9) with modifications (38). Free-floating sections were blocked in PBS containing 5% goat serum, 0.1% BSA, 0.05% Triton-X100, 1 mM NaF for 1 hr at room temperature and incubated with anti-phospho-AMPKa (a1 and a2) antibody (1:100)

or anti-phospho- CREB antibody (1:500, Cell Signaling) in PBS containing 1% goat serum, 0.1% BSA, 0.05% Triton-X100, 1 mM NaF overnight at 4 °C. Signal was visualized by Vectastain ABC kit (Vector).

In situ hybridization. Anti-sense DIG-labeled NPY riboprobe was generated from a plasmid containing the NPY gene (XM004941). Hybridization and washing were performed as described (9). For double fluorescent in situ hybridization, DIG-labeled riboprobe was generated from plasmid containing AMPKa2 gene (pEBGa2, a gift from L. A. Witters) for AMPKa2 (FITC) and biotin-labeled riboprobe was used for NPY (Texas Red). Sheep FITC-conjugated anti-DIG antibody (1:50, Roche) was incubated in TNB buffer (100mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.5% blocking reagent) for FITC detection. Streptavidin-Texas Red (1:50, Amersham Pharmacia), rabbit anti-Texas Red antibody (1: 50, Molecular Probes), goat biotin-conjugated anti-rabbit IgG antibody (1:50, Santa Cruz Biotechnology) and streptavidin-Texas Red (1:30) were incubated serially in TNB buffer for Texas Red detection.

Analysis and quantification of images. Images of in situ hybridization and immunohistochemistry were visualized using an Axiocam HRc digital camera (Carl Zeiss) and images were acquired using Improvision Openlab software, and quantified by NIH Image program (Macro). Statistical analysis. All values are presented as mean±SEM. Data were analyzed by one-way ANOVA or t-test.

Example 1

Feeding behavior is changed by C75, AICAR or compound C treatment.

Mice were implanted with intracerebroventricular (i.c.v.) cannulae to measure food intake after dark onset administration of C75 (Fig. 1a). All mice had access to food ad libitum during the 24 hr cycle. C75 significantly reduced food intake during the 1-3 and 3-24 hr time intervals in a dose-dependent manner (Fig.1a). Injection of 5 and 10 mg of C75 caused a 20.3% ($p<0.05$) and 37.7% ($p<0.01$) reduction in food intake over 24 hr, respectively. The 10 μg dose also produced a reduction in body weight (Fig. 1d). These results indicate that C75 reduces food intake via central mechanism(s).

AICAR (5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside), a compound that stimulates AMPK activity, is taken up into cells and phosphorylated to form ZMP (24), which mimics the effects of AMP on AMPK activation (25). We determined the time course of action of AICAR, as its effect may be transient, since it is further metabolized (26). In contrast to the feeding inhibition produced by C75, i.c.v., administration of AICAR increased food intake. A dose of 3 μg increased food intake to 230% ($p<0.01$) within 1 hr, 135% ($p<0.01$) at 3-24 hr and total 24 hr food intake was increased to 130% of control ($p<0.05$) (Fig. 1b). Despite this increase in food intake, this single dose of AICAR has no significant effect on bodyweight (Fig. 1d). Bodyweight does not always change in proportion to food intake (27). A previous report noted that chronic subcutaneous injection of AICAR (1 g/kg bodyweight) for 4 weeks had no impact on either food intake or bodyweight (28), but that there was a reduction in fat pad mass and an increase in liver mass. Thus, i.c.v. administration of a single dose of AICAR may have an effect on the mass of these peripheral tissues, such that bodyweight does not change despite increased food intake.

To confirm the effect of AMPK on food intake, we used compound C, which is a selective AMPK inhibitor (29,30). The i.c.v. injection of 5 mg compound C caused a 36.2%, 37.8% and 35.6% reduction in food intake at 0-1, 3-24 hr and over 24 hr, respectively (Fig. 1c). This dosage of compound C led to a weight loss (Fig. 1d). Interestingly, as with the stimulatory effect of AICAR on feeding, the inhibitory effect of compound C on feeding was profound at 0-1 hr and 3-24 hr. The intraperitoneal (i.p.) injection of compound C also had a similar reduction in food intake (Fig. 1e), showing that a higher dose (30 mg/kg bodyweight) decreased food intake during all time intervals (27.4%, 3.68%, 65.7% and 57.8% of control during 0-1, 1-3, 3-24 hr and total, respectively). Even though AICAR or compound C may have additional cellular effects that cannot be excluded, the opposite results on food intake obtained using an AMPK activator and inhibitor supports the hypothesis that AMPK is involved in feeding behavior. We determined the time course of action of the i.p. C75 administration, with the intention of utilizing this route of administration for C75 in further experiments designed to compare the central and peripheral effects of C75 on the change in AMPK activation, and to combine C75 and AICAR treatments. Administration of i.p. C75 (10 mg/kg bodyweight) caused a dramatic decrease in food intake during all intervals measured (8.3%, 23.3%, and 30.1% of control during 0-1, 1-3, and 3-24 hr, respectively) (Fig. 1f). Total 24 hr-food consumption was significantly reduced to 26.3% of control ($p<0.001$). The effect of C75 on food intake was more pronounced and lasted longer than that of compound C. The greater magnitude of the effect following peripheral administration of C75 on food intake may reflect the larger dose that can be administered

via this route, or an additional peripheral effect, compared to the i.c.v. route of administration.

Collectively, these results demonstrate that C75 and compound C (administered either i.c.v. or i.p.) produce opposite effects on food intake over similar time courses compared to i.c.v. administration of AICAR.

Example 2

C75 decreases the phosphorylation of hypothalamic AMPK.

The hypothalamus plays an important role in monitoring energy balance and integrating peripheral signals that affect food intake (1, 2). Although the expression of AMPK in brain has been reported (31, 32), its function in the brain was previously unknown. C75 inhibits FAS and stimulates carnitine palmitoyl transferase-1 (CPT-1), the enzyme that imports palmitate into the mitochondrion for β-oxidation (8). Both of these actions may signal a positive energy balance in neurons of the hypothalamus, which may inactivate hypothalamic AMPK. To examine the effect of C75 on hypothalamic AMPK activity, we determined the effect of C75 treatment on the level of phosphorylation of the a catalytic subunit (18) of AMPK (pAMPKα) in the hypothalamus, which correlates with its activity (Fig. 2).

Mice received vehicle, 5 μg, or 10 mg of C75 i.c.v., and the levels of hypothalamic pAMPKα were determined by Western blot. The level of AMPKα (α1 and α2 subunits) served as a loading control. Compared to levels of pAMPKα in vehicle-treated control animals, C75 reduced the levels of pAMPKα (α1 and α2) in the hypothalamus at 30 min and 3 hr three- and six-fold, respectively (Fig. 2a,b). As seen with central administration of C75, i.p. injection of C75 (10 mg/kg body weight)

significantly reduced the levels of pAMPKα in the hypothalamus at 30 min and 3 hr (Fig. 2c,d). In contrast, C75 had little effect on pAMPKα levels in the liver 30 min after administration, but increased pAMPKα levels at 3 hr (Fig. 2e,f). These results demonstrated that C75 rapidly decreased AMPK activity in the hypothalamus. The decrease in hypothalamic pAMPKα levels could result from the metabolic changes that occur as a result of FAS inhibition, which would diminish energy expenditure and signal a favorable energy balance. These results also indicate that the phosphorylation of AMPK is regulated differently in the hypothalamus than in the liver in response to C75. This difference most likely reflects differences between metabolic pathways, or the flux through those pathways, found in neurons and in liver. By 3 hr, the decreased food intake seen with C75 treatment may signal an energy poor state in liver (Fig. 2e), leading to AMPK activation, indicating an attempt to preserve energy levels through the stimulation of fatty acid oxidation, for example.

C75 decreases the fasting-induced phosphorylation of hypothalamic AMPK

It has been shown that the activity of AMPK is elevated in fasted rat liver (33). To investigate whether hypothalamic AMPK is responsive to fasting, the level of pAMPKα was monitored after withdrawal of food at the onset of dark cycle in mice fed ad libitum. There was no change in pAMPKα levels within 3 hr of food withdrawal (Fig. 3a,b). However, fasting for 24 hr resulted in a two-fold stimulation in the level of hypothalamic pAMPKα (Fig. 3a,b). While one report (34) noted no difference in AMPK activity between dark and light cycles in rats fed ad libitum, only one time point (6 hr) was investigated, without correlation to feeding profile in the interval before this

measurement was made. Our results suggest that the activation of hypothalamic AMPK could be involved in the fasting-induced stimulation of food intake.

We next examined whether C75 could reduce AMPK phosphorylation in the setting of fasting, when AMPK phosphorylation is increased. This is important in establishing a link between C75-induced FAS inhibition and AMPK activity, as C75 does inhibit feeding even in fasted mice (4, 8). After 24 hr of fasting, either vehicle (RPMI) or C75 was administrated i.p., and the levels of hypothalamic pAMPKa were determined. C75 treatment profoundly reduced the level of pAMPKa compared to that of control (Fig. 3c.d). Given our observation that C75 suppresses food intake even in fasted mice, the ability of C75 to reduce the levels of pAMPKα in fasted mice supports that C75 might inhibit feeding by an AMPK-mediated mechanism.

C75 increases the hypothalamic neuronal ATP level

It has been shown that C75 increases ATP levels in 3T3-L1 adipocytes (8) and even in primary cortical neurons (35). Since an increase in the AMP/ATP ratio is known to activate AMPK (15, 16), we hypothesized that a C75-induced increase in hypothalamic ATP levels could contribute to a decrease in AMP/ATP, resulting in reduced hypothalamic AMPK activity. Treatment of primary cultures of hypothalamic neurons with 40 mg/ml C75 led to a significant increase in neuronal ATP levels to 118 and 128% of control at 30 min and 2 hr, respectively (Fig. 4a). C75 treatment caused a similar change in ATP levels in primary cortical neurons (35), producing a decrease in the ratio of AMP/ATP and inactivation of AMPK. Therefore, It is likely that an increase in ATP caused by C75 also contributed to the decrease in AMPK activity in the hypothalamus.

AICAR reverses C75's anorexic effect and increases the phosphorylation of hypothalamic AMPK

To determine whether AICAR could reverse the C75-induced decrease in food intake, we treated mice 1 hr before the onset of dark cycle with either vehicle or C75 (5 mg/kg bodyweight) i.p., followed 1 hr later by an i.c.v. injection of vehicle or AICAR (3 mg) (Fig. 4b). C75 reduced food intake at 1 hr to 37.5% of control (RPMI/saline) ($p<0.01$). In contrast, AICAR treatment increased food intake at 1 hr to 346% of the amount of C75/saline treatment ($p<0.001$). AICAR treatment reversed the C75-induced anorexia, resulting in food intake that was similar to that of control vehicle-treated mice. The effect of AICAR on C75-treated mice was of limited duration, consistent with the metabolism of AICAR (26). The lack of an effect on food intake during the 3-24 hr time interval may represent the net effect of the opposing actions of C75 and AICAR. If the reversal of C75-mediated anorexia by AICAR involves alteration of AMPK activity, ICAR should similarly reverse the decrease in the level of hypothalamic pAMPKα that occurs with C75 treatment. Ad libitum fed mice received an i.p. injection followed by an i.c.v. injection 1 hr later as follows: i.p. RPMI and i.c.v. saline; i.p. RPMI and i.c.v. AICAR; i.p. C75 and i.c.v. saline; and i.p. C75 and i.c.v. AICAR (Fig. 4c). Hypothalamic tissues were prepared for Western blot 30 min after the i.c.v. injection (Fig. 4c,d). A low level of pAMPKa was detected in vehicle-treated mice, which was increased in AICAR-treated animals (Fig. 4c,d). Mice that received C75 i.p. and saline i.c.v. displayed a profound decrease in pAMPKa levels.

AICAR treatment following C75 treatment completely reversed the C75-induced decrease in hypothalamic pAMPKα levels. Sub-threshold doses would have been used with only behavioral data, but the fact that AICAR prevented the C75 induced changes in both behavior and the status of AMPK phosphorylation support a common site of action for the effects of C75 and AICAR. These results indicate that AICAR restores both C75-induced anorexia and the C75-induced suppression of AMPK activity.

C75 alters pAMPK, pCREB and NPY expression in arcuate neurons.

AMPK acutely regulates cellular metabolism and chronically regulates gene expression (36). To ascertain whether the changes in the phosphorylation status of AMPK in the hypothalamus reflected the level of pAMPKα in the arcuate nucleus, we performed immunohistochemistry for pAMPKα using coronal brain sections containing the arcuate nucleus (Fig. 5a1-3). pAMPKα was detected in the arcuate nucleus of mice fed ad libitum (Fig. 5a1), and immunostaining was successfully blocked by preabsorbing with phospho-AMPKa peptide (data not shown). Compared to control, pAMPKaimmunoreactivity was increased to 171% of control in the arcuate nucleus of mice fasted for 24 hr (Fig. 5a3). pAMPKa-immunoreactivity was reduced in C75-treated mice to 12% of control, even in the setting of reduced food intake (Fig. 5a2). These changes are consistent with our Western blot data (Fig. 2a,c), and confirm that C75 reduces pAMPKα levels in the arcuate nucleus.

We have previously demonstrated by Northern blot analysis that C75 decreased hypothalamic NPY expression (4, 9). We next investigated whether the decreases in pAMPKa in the arcuate correlated with changes in NPY that occur with C75

treatment. NPY expression in neurons within the arcuate nucleus was determined in control, C75- treated, and fasted mice (Fig. 5a4-6). Consistent with previous Northern blot analysis of hypothalamic tissues (9), NPY mRNA expression was down regulated in the arcuate nucleus of C75-treated mice to 66% of control (Fig. 5a5) and up regulated in fasted mice to 140% of control (Fig. 5a6). It has been shown that the cAMP-CREB pathwaymediates NPY expression under fasted conditions (37, 38), suggesting that leptin modulates NPY gene expression through this pathway (38). To elucidate the pathways involved in the down-regulation of NPY that occurs with C75 treatment, we determined the level of pCREB in the arcuate nucleus (Fig. 5a7-9). As previously reported (38), 24 hr fasting increased pCREB immunoreactivity in the arcuate nucleus to 197% of control (Fig. 5a9). In contrast, C75 decreased the level of pCREB to 39% of control (Fig. 5a8), consistent with the hypothesis that the decrease in NPY gene expression caused by C75 may be mediated by a decreased level of pCREB. To clarify the co-localization of AMPK and NPY in the arcuate nucleus, double in situ hybridization was performed (Fig.5b). A subpopulation of neurons in the arcuate nucleus that expressed AMPKa2 mRNA also expressed NPY mRNA (Fig. 5b). It is known that NPY and CREB co-localize to neurons in the arcuate nucleus (38). These results indicate that AMPK, NPY, and CREB are co-expressed in a subpopulation of neurons within the arcuate nucleus, and support the hypothesis that AMPK may modulate CREB phosphorylation to affect NPY expression.

In contrast to C75, AICAR had the opposite effect (Fig. 5c,d). Thus, consistent with our findings that AICAR stimulated feeding, AICAR significantly increased hypothalamic NPY expression 20 hrs after i.c.v. administration (Fig. 5c). The increase in NPY expression seen with AICAR treatment may mediate the stimulation of

food intake seen at later times (3-24 hr) in Fig. 1b. Since no change in NPY expression with AICAR treatment was detected within 5 hr (data not shown), it appears that the earlier change in feeding (0-1 hr) is mediated by NPY gene expression-independent mechanism. AICAR also increased pCREB level in the arcuate up to 231% of control (Fig. 5d), which supports that AMPK may modulate CREB phosphorylation.

Detailed Description of the Figures

Figure 1. Food intake is affected by C75, AICAR or compound C.

(a) BALB/c male mice (n=7-9) received an i.c.v. injection of either 2.5 μl of RPMI with or without C75 (5 or 10 μg), and food intake was monitored as described in Methods.

(b) Mice (n=4-10) received an i.c.v. injection of 2.5 μl of saline with or without AICAR (1 or 3 μg), and food intake was monitored.

(c) Food intake was measured from mice (n=7-8) received an i.c.v. injection of 2.5 μl of saline with or without compound C (2 or 5 μg).

(d) Changes in bodyweight 24 hr after i.c.v. injection of C75, AICAR or compound C (n=4-10).

(e) Two hundred μl of vehicle (saline) or saline containing compound C (10 or 30 mg/kg bodyweight) was administered i.p. to mice (n=4-7).

(f) Two hundred μl of vehicle (RPMI) or RPMI containing C75 (10 mg/kg bodyweight) was administered i.p. to mice (n=4-9). Data were combined from three experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, compared to vehicle RPMI or saline treatment.

Figure 2. C75 treatment reduces the phosphorylation of hypothalamic AMPKα.

(a) Levels of phosphorylated AMPKα (α1 and α2) and total AMPKa (α1 and α2) were visualized by Western blot analysis in extracts of hypothalamus at various times after i.c.v. injection of C75 (5 or 10 mg) at onset of dark cycle.

(c,e) Levels of phosphorylated AMPKa and total AMPKa from hypothalamus (c) or liver (e) after i.p. injection of C75 (10 mg/kg bodyweight). Tissue samples were prepared 1 hr after the i.p. injection. (cand e). Quantification from Western blot (Fig. 2a,c and e) epresents the fold-difference in ratio (phosphorylated AMPKα/ total AMPKα) compared to control. The sensitivity of signal detection for phosphorylated AMPKa is 100-fold higher than total AMPKα.

Figure 3. C75 also reduces the fasting-induced phosphorylation of hypothalamic AMPKa.

(a) Levels of phosphorylated AMPKα and total AMPKα were visualized in the hypothalamus from control (ad libitum access to food) and fasted mice. Food was withdrawn at onset of dark cycle (0 hr) over 24 hr, and the levels of phosphorylated and total AMPKa was determined at 0 hr, 3 hr and 24 hr after fasting.

(b) The graphs show the fold-difference from quantification of Western blot (Fig. 3a).

(c) Levels of phosphorylated and total hypothalamic AMPKa were determined in mice that were fasted for 24 hr and then received an i.p. injection of RPMI with or without C75 (10 mg/kg bodyweight). Tissue samples were prepared 1 hr after the i.p. injection.

(d) The graphs show the fold-difference from quantification of Western blot (Fig. 3c).

Figure 4. C75 alters ATP level of hypothalamic neuron and AICAR reverses both C75-induced anorexia and reduction in pAMPKa levels.

(a) Primary hypothalamic neurons were treated with 20 or 40 mg/ml of C75 for 30 min and 2 hr. ATP levels were evaluated by luminescence and represented as a % of untreated controls (-). Data were combined from three independent experiments. **, $p<0.01$ compared to untreated control.

(b) Food intake was determined for mice (n=10- 12) that received i.p. C75 (5 mg/kg bodyweight) followed by injection i.c.v. AICAR (3 mg) 1 hr later. Food intake was monitored at same time intervals as in Fig. 1a. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared to RPMI/saline or C75/saline.

(c) Levels of hypothalamic phosphorylated and total of AMPKa were determined by Western blot in mice that received i.p. RPMI/i.c.v. saline, i.p.RPMI/i.c.v. AICAR, i.p. C75/i.c.v. saline or i.p. C75/i.c.v. AICAR using the same dosages as in Fig. 4b. (d) The graphs show the fold-difference from quantification of Western blot (Fig. 4c).

Figure 5. C75 affects pAMPKa, NPY, and pCREB expression in the arcuate nucleus.

(a) Immunohistochemistry of pAMPKa (1,2,3), in situ hybridization of NPY (4,5,6) and immunohistochemistry of pCREB (7,8,9) in the arcuate nucleus was performed using coronal brain sections from control, C75-treated (24 hr) and fasted (24 hr) mice.

(b) Colocalization of AMPKa2 (FITC) and NPY (Texas Red) in arcuate nucleus neurons by double fluorescent in situ hybridization.

(c) mRNA level of hypothalamic neuropeptides was determined by Northern analysis from mice (n=4 each) that received i.c.v. saline or AICAR (3 mg) 20 hr after injection. ***, $p<0.001$ compared to saline control.

(d) The arcuate pCREB levels (under dashed line) were shown from mice that received i.c.v. saline or AICAR (3 mg) 20 hr after injection. (e) A model for C75-induced changes in energy flux that alter AMPK activity to modulate CREB-NPY pathway signaling in the arcuate nucleus.

Claims

1. A method for regulating food intake by administering a compound which targets the activity of AMPK.

2. The method of claim 1, wherein the AMPK is hypothalmic AMPK.

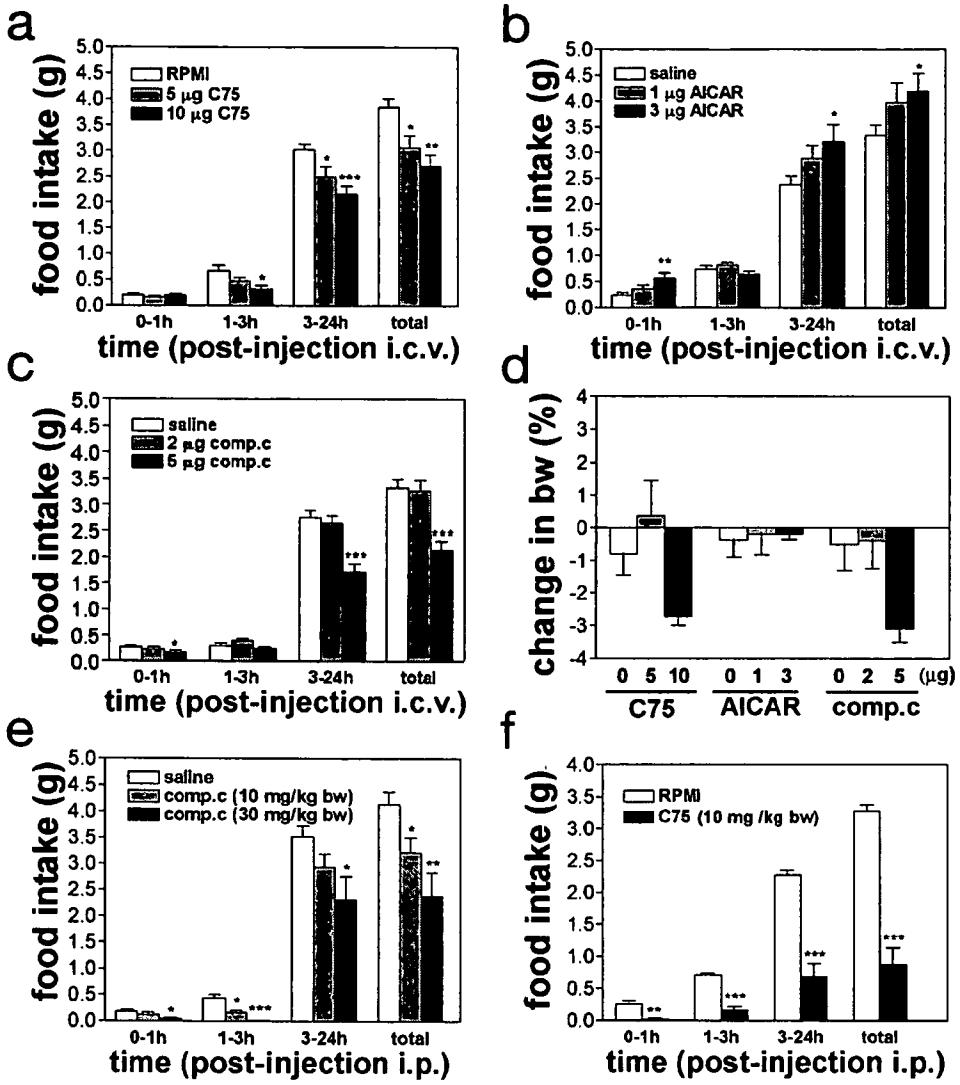
3. The method of claim 1, wherein the compound is not C75.

4. The method of claim 1, wherein the compound is C75.

References

1. Wilding, J. P. (2002) Diabet Med 19, 619-27.
2. Schwartz, M., Woods, S., Porte D, J., Seeley, R. & Baskin, D. (2000) Nature 404, 661-671.
3. Woods, S. C., Schwartz, M. W., Baskin, D. G. & Seeley, R. J. (2000) Annu Rev Psychol 51, 255-77.
4. Loftus, T., Jaworsky, D., Frehywot, G., Townsend, C., Ronnett, G., Lane, M. & Kuhajda, F. (2000) Science 288, 2379-2381.
5. Clegg, D. J., Wortman, M. D., Benoit, S. C., McOsker, C. C. & Seeley, R. J. (2002) Diabetes 51, 3196-201.
6. Kumar, M. V., Shimokawa, T., Nagy, T. R. & Lane, M. D. (2002) Proc Natl Acad Sci U S A 99, 1921-5.
7. Shimokawa, T., Kumar, M. V. & Lane, M. D. (2002) Proc Natl Acad Sci U S A 99, 66-71.
8. Thupari, J. N., Landree, L. E., Ronnett, G. V. & Kuhajda, F. P. (2002) Proc Natl Acad Sci U S A 99, 9498-502.
9. Kim, E. K., Miller, I., Landree, L. E., Borisy-Rudin, F. F., Brown, P., Tihan, T., Townsend, C. A., Witters, L. A., Moran, T. H., Kuhajda, F. P. & Ronnett, G. V. (2002) Am J Physiol Endocrinol Metab 283, E867-79.
10. da Silva Xavier, G., Leclerc, I., Salt, I. P., Doiron, B., Hardie, D. G., Kahn, A. & Rutter, G. A. (2000) Proc Natl Acad Sci U S A 97, 4023-8.
11. Marsin, A. S., Bertrand, L., Rider, M. H., Deprez, J., Beauloye, C., Vincent, M. F., Van den Berghe, G., Carling, D. & Hue, L. (2000) Curr Biol 10, 1247-55.
12. Mu, J., Brozinick, J. T., Jr., Valladares, O., Bucan, M. & Birnbaum, M. J. (2001) Mol Cell 7, 1085-94.
13. Musi, N., Fujii, N., Hirshman, M. F., Ekberg, I., Froberg, S., Ljungqvist, O., Thorell, A. & Goodyear, L. J. (2001) Diabetes 50, 921-7.
14. Ponticos, M., Lu, Q. L., Morgan, J. E., Hardie, D. G., Partridge, T. A. & Carling, D. (1998) Embo J 17, 1688-99.
15. Hardie, D. G. & Carling, D. (1997) Eur J Biochem 246, 259-73.
16. Winder, W. W. (2001) J Appl Physiol 91, 1017-28.
17. Hardie, D. G., Salt, I. P., Hawley, S. A. & Davies, S. P. (1999) Biochem J 338, 717-22.
18. Hawley, S. A., Davison, M., Woods, A., Davies, S. P., Beri, R. K., Carling, D. & Hardie, D. G. (1996) J Biol Chem 271, 27879-87.
19. Kurth-Kraczek, E. J., Hirshman, M. F., Goodyear, L. J. & Winder, W. W. (1999) Diabetes 48, 1667-71.
20. Corton, J. M., Gillespie, J. G. & Hardie, D. G. (1994) Curr Biol 4, 315-24.
21. Corton, J. M., Gillespie, J. G., Hawley, S. A. & Hardie, D. G. (1995) Eur J Biochem 229, 558-65.
22. Hardie, D. G. (1992) Biochim Biophys Acta 1123, 231-8.
23. Minokoshi, Y., Kim, Y.-B., Peroni, O., Fryer, L. G., Muller, C., Carling, D. & Kahn, B. (2002) Nature 415, 339-343.
24. Sabina, R. L., Patterson, D. & Holmes, E. W. (1985) J Biol Chem 260, 6107-14.
25. Sullivan, J. E., Brocklehurst, K. J., Marley, A. E., Carey, F., Carling, D. & Beri, R.

K. (1994) FEBS Lett 353, 33-6.
26. Kemp, B. E., Mitchelhill, K. I., Stapleton, D., Michell, B. J., Chen, Z. P. & Witters, L. A. (1999) Trends Biochem Sci 24, 22-5.
27. Abu-Elheiga, L., Matzuk, M. M., Abo-Hashema, K. A. & Wakil, S. J. (2001) Science 291, 2613-6.
28. Winder, W. W., Holmes, B. F., Rubink, D. S., Jensen, E. B., Chen, M. & Holloszy, J. O. (2000) J Appl Physiol 88, 2219-26.
29. Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M. F., Goodyear, L. J. & Moller, D. E. (2001) J Clin Invest 108, 1167-74.
30. Lee, M., Hwang, J. T., Lee, H. J., Jung, S. N., Kang, I., Chi, S. G., Kim, S. S. & Ha, J. (2003) J Biol Chem 278, 39653-61.
31. Turnley, A. M., Stapleton, D., Mann, R. J., Witters, L. A., Kemp, B. E. & Bartlett, P. F. (1999) J Neurochem 72, 1707-16.
32. Culmsee, C., Monnig, J., Kemp, B. E. & Mattson, M. P. (2001) J Mol Neurosci 17, 45-58.
33. Witters, L. A., Gao, G., Kemp, B. E. & Quistorff, B. (1994) Arch Biochem Biophys 308, 413-9.
34. Davies, S. P., Helps, N. R., Cohen, P. T. & Hardie, D. G. (1995) FEBS Lett 377, 421-5.
35. Landree, L. E., Hanlon, A. L., Strong, D. W., Rumbaugh, G., Miller, I. M., Thupari, J. N., Connolly, E. C., Huganir, R. L., Richardson, C., Witters, L. A., Kuhajda, F. P. & Ronnett, G. V. (2003) J Biol Chem in press.
36. Hardie, D. G. & Hawley, S. A. (2001) Bioessays 23, 1112-9.
37. Sheriff, S., Chance, W. T., Fischer, J. E. & Balasubramaniam, A. (1997) Mol Pharmacol 51, 597-604.
38. Shimizu-Albergine, M., Ippolito, D. L. & Beavo, J. A. (2001) J Neurosci 21, 1238-46.
39. Hardie, C., and Carlson (1998) The AMP-Activated /SNF1 Protein Kinase Subfamily: Metabolic Sensors of the Eukaryotic Cell.
40. Sprenkle, A. B., Davies, S. P., Carling, D., Hardie, D. G. & Sturgill, T. W. (1997) FEBS Lett 403, 254-8.
41. Salt, I., Celler, J. W., Hawley, S. A., Prescott, A., Woods, A., Carling, D. & Hardie, D. G. (1998) Biochem J 334 (Pt 1), 177-87.
42. Leff, T. (2003) Biochem Soc Trans 31, 224-7.
43. Obici, S., Feng, Z., Arduini, A., Conti, R. & Rossetti, L. (2003) Nat Med 9, 756-61.
44. Wortman, M. D., Clegg, D. J., D'Alessio, D., Woods, S. C. & Seeley, R. J. (2003) Nat Med 9, 483-5.
45. Winder, W. W. (2000) Diabetes Technol Ther 2, 441-8.
46. Fraley, M. E., Rubino, R. S., Hoffman, W. F., Hambaugh, S. R., Arrington, K. L., Hungate, R. W., Bilodeau, M. T., J.;, T. A., Rutledge, R. Z., Kendall, R. L., McFall, R. C., Huckle, W. R., E.;, C. K. & Thomas, K. A. (2002) Bioorg. Med. Chem. Lett. 12, 3537-3541.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food intake (g)
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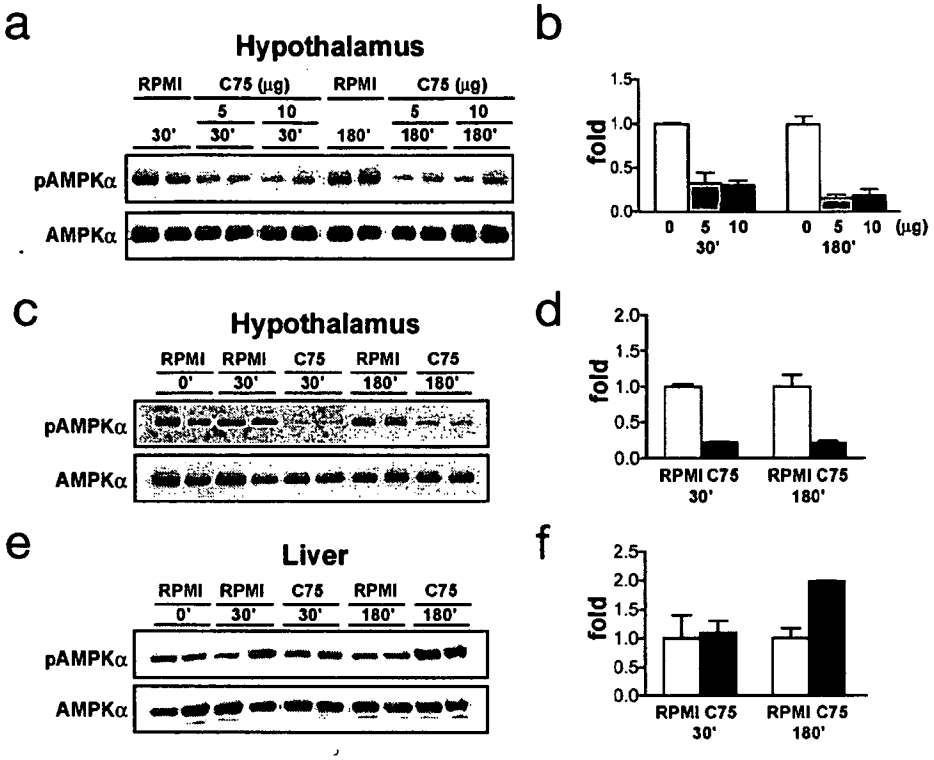
10 µg C75
0-1h
1-3h
3-24h
total
time (post-injection i.c.v.)
b
food intake (g)
saline
1 µg AICAR
3 µg AICAR
time (post-injection i.c.v.)
c
food intake (g)
saline
2 µg comp.c
5 µg comp.c
time (post-injection i.c.v.)
d
change in bw (%)
0 5 10
0 1 3
0 2 5 (µg)
C75
AICAR
comp.c
e
food intake (g)
saline
comp.c (10 mg/kg bw)
comp.c (30 mg/kg bw)
time (post-injection i.p.)
f
food intake (g)
RPMI
C75 (10 mg /kg bw)
time (post-injection i.p.)

Fig. 1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Hypothalamus
RPMI
C75 (μg)
RPMI
C75 (μg)
5
10
5
10
30'
30'
30'
180'
180'
180'
pAMPKα
AMPKα
b
fold
1.5
1.0
0.5
0.0
0
5
10
0
5
10
(μg)
30'
180'
c
Hypothalamus
RPMI
RPMI
C75
RPMI
C75
0'
30'
30'
180'
180'
pAMPKα
AMPKα
d
fold
2.0
1.5
1.0
0.5
0.0
RPMI C75
RPMI C75
30'
180'
e
Liver
RPMI
RPMI
C75
RPMI
C75
0'
30'
30'
180'
180'
pAMPKα
AMPKα
f
fold
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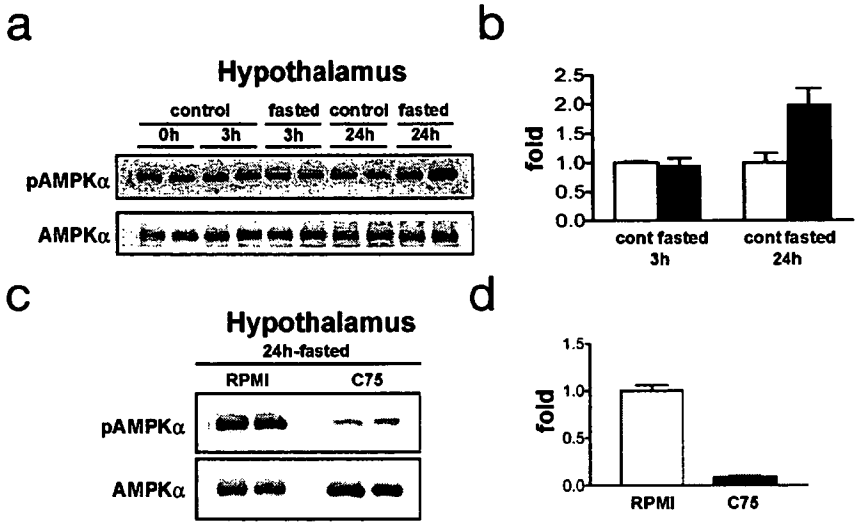
1.0
0.5
0.0
RPMI C75
RPMI C75
30'
180'

Fig. 2


a
Hypothalamus
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fasted
control
fasted
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3h
3h
24h
24h
pAMPKα
AMPKα
b
fold
2.5
2.0
1.5
1.0
0.5
0.0
cont
fasted
cont
fasted
3h
24h
c
Hypothalamus
24h-fasted
RPMI
C75
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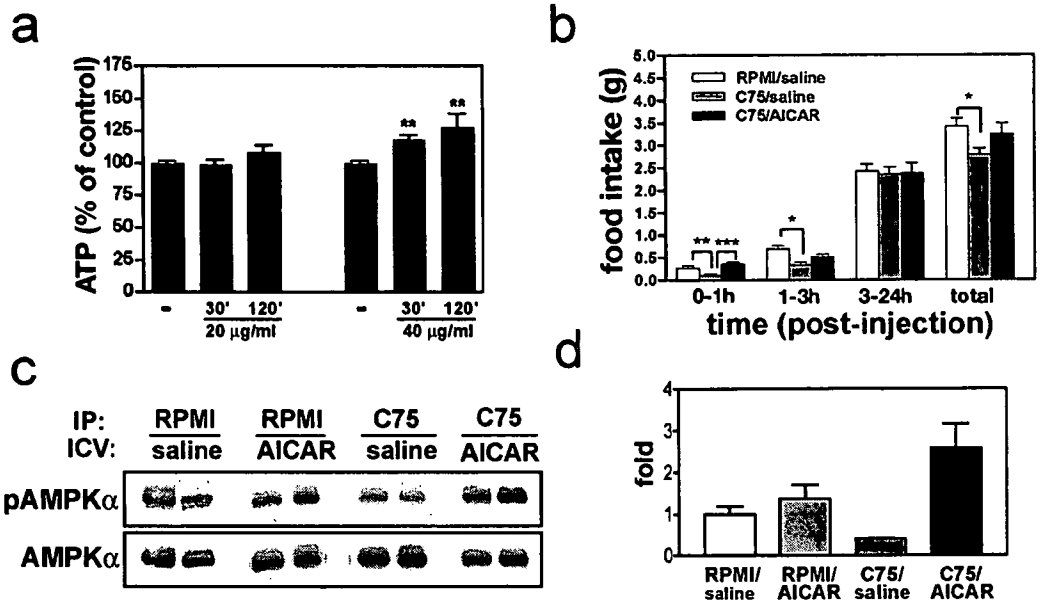
AMPKα
d
fold
1.5
1.0
0.5
0.0
RPMI
C75

Fig. 3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ATP (% of control)
175
150
125
100
75
50
25
0
-
30'
120'
20 µg/ml
-
30'
120'
40 µg/ml
**
**
b
food intake (g)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
RPMI/saline
C75/saline
C75/AICAR
**
***
*
*
0-1h
1-3h
3-24h
total
time (post-injection)
c
IP:
ICV:
RPMI
saline
RPMI
AICAR
C75
saline
C75
AICAR
pAMPKα
AMPKα
d
fold
4
3
2
1
0
RPMI/
saline
RPMI/
AICAR
C75/
saline
C75/
AICAR

Fig. 4

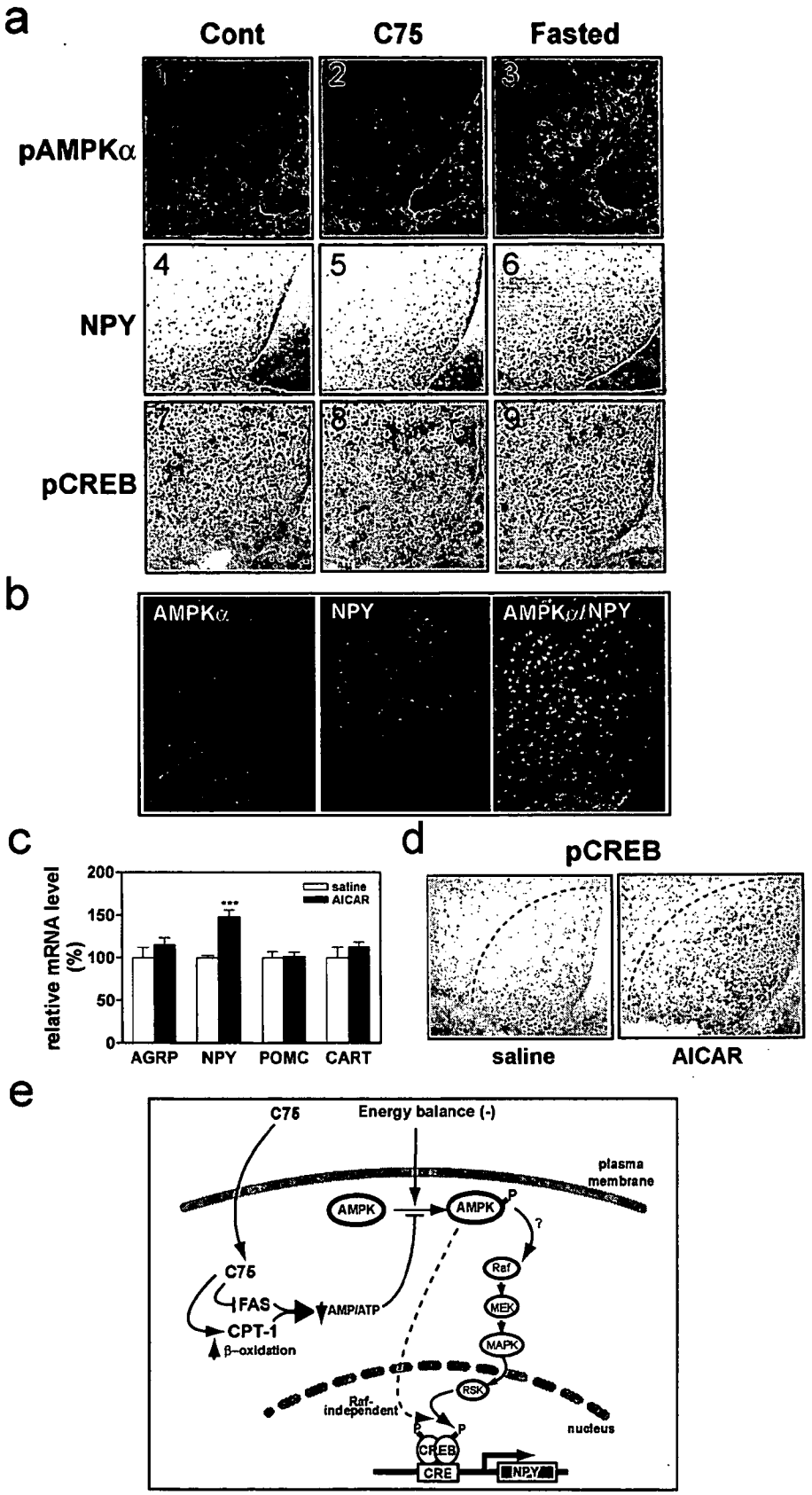

Fig. 5